FINAL REPORT

GRANT #: N00014-98-1-0657

PRINCIPAL INVESTIGATOR: Dr. Frances H. Arnold

INSTITUTION: California Institute of Technology

GRANT TITLE: Expression and Directed Evolution of Peroxidase Enzymes

AWARD PERIOD: 1 May 1998 – 30 April 2000

OBJECTIVE: The overall goal of this research is to develop strategies for the evolution of novel functions in proteins. The main objective of this project has been to realize the functional expression of eukaryotic peroxidases in facile recombinant hosts (bacteria and yeast), which is a prerequisite to tailoring these enzymes for industrial and Naval applications. We have improved the folding and expression of horseradish peroxidase in recombinant hosts by directed evolution.

APPROACH: When expressed in the recombinant organisms such as *E. coli* or yeast, these enzymes often fail to fold into the active form. Instead, the polypeptides accumulate in inactive aggregates. We have sought to improve the folding and expression of horseradish in recombinant hosts by modifying its sequences using "directed evolution". Our goal is to search systematically for mutations in the genes that can facilitate folding of the polypeptides in the recombinant host environments, without compromising catalytic activity.

ACCOMPLISHMENTS: We developed a high throughput screen for HRP activity in complex media and used this to identify HRP mutants that are functionally expressed in both bacteria (E. coli) and yeast (*S. cerevisiae*). Through three rounds of directed evolution by random point mutagenesis and screening, we obtained a 40-fold increase in total HRP activity in the *S. cerevisiae* culture supernatant compared to wild type, as measured on ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid). Genes from wild type and two high-activity clones were expressed in *P. pastoris*, where the total ABTS activity reached 6000 units/L in shake flasks. The mutants show up to 5.4-fold higher specific activity towards ABTS and 2.3-fold higher towards guaiacol. Similar results have been obtained in E. coli, from which the enzyme is also secreted into the medium. *E. coli*-expressed HRP was also used to screen large libraries of cytochrome P450 mutants by combining the P450-catalyzed hydroxylation with a peroxidase-catalyzed oxidative coupling reaction *in vivo*.

CONCLUSIONS: It had been reported that horseradish peroxidase (HRP), a glycosylated heme peroxidase, fails to express in active form in *E. coli* and yeast. We proposed that we could find mutations in the HRP gene or regulatory elements that allow functional expression of this enzyme. We have shown this to be the case.

SIGNIFICANCE: Peroxidases are useful enzymes for organic synthesis, bioremediation and biosensor applications. However, the naturally-occurring enzymes exhibit properties that limit their practical applications, including sensitivity to peroxide, poor stability and low activity towards nonnatural substrates or in nonnatural environments. We have succeeded in expressing HRP in bacterial and yeast hosts suitable for directed evolution. These systems will now allow us to optimize HRP for specific applications by directed

evolution. We have also clearly demonstrated that these, and presumably other, enzymes can indeed be expressed in *E. coli* and yeast.

PATENT INFORMATION:

Filed U.S. patent application "Expression of Functional Eukaryotic Proteins, Ser. No. 09/247,232, February 9, 1999. Filed continuation-in-part March 27, 2000.

AWARD INFORMATION: Frances Arnold was elected to National Academy of Engineering, January 2000. Jeffrey Moore, a former Ph.D. student in this group, was awarded a major international biotechnology prize, the Lonza Centenary Biotechnology Prize, for his work in this laboratory on directed enzyme evolution, June 1998.

PUBLICATIONS AND ABSTRACTS:

1. Giver, L. and F. Arnold (1998) Combinatorial protein design by *in vitro* recombination. Current Opinion in Chemical Biology **2**:335-338.

2. Arnold F. (1998) When blind is better: protein design by evolution. Commentary in Nature Biotechnology **16**:617.

3. Arnold, F. A. (1998) Design by directed evolution. Accounts of Chemical Research **31**:125-131.

4. Joo, H., L. Lin and F. H. Arnold (1999) Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation. Nature **399**:670-673.

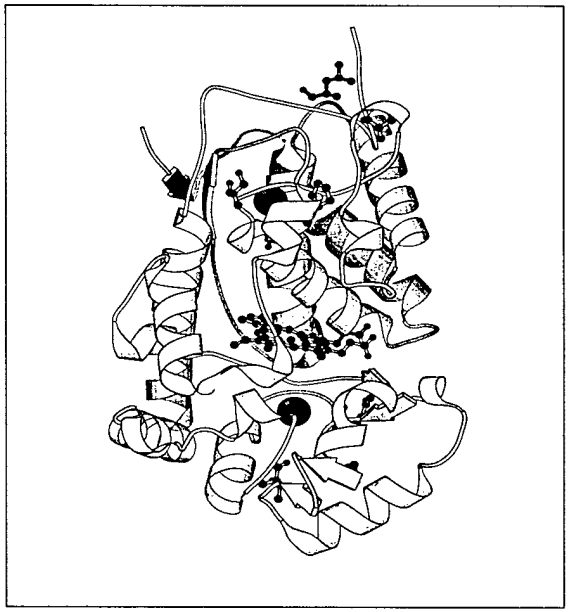
5. Affholter, J. and F. H. Arnold (1999) Engineering a revolution. Chemistry in Britain **35**:48-51.

6. Schmidt-Dannert, C. and F. H. Arnold (1999) Directed evolution of industrial enzymes. Trends in Biotechnology **17**:135-136.

7. Morawski B., Z. Lin, P. Cirino, H. Joo and F. H. Arnold (2000) Functional expression of horseradish peroxidase in *Saccharomyces cerevisiae and Pichia pastoris*, Protein Engineering **13**:377-384.

Expression and directed evolution of peroxidase enzymes

Frances Arnold, Caltech



Objective:

- *Obtain functional expression of horseradish peroxidase (HRP) in E. coli and S. cerevisiae*

Approach:

- *Random mutation of HRP gene and high throughput screening for activity for several generations*

Accomplishments:

- *40-fold increase in total activity in S. cerevisiae*
- *Expression in Pichia pastoris demonstrated*
- *Peroxide-stable and thermostable mutants obtained*

Transitions:

- *Technology has been licensed to Maxygen, Inc.*

REPORT DOCUMENTATION PAGE

Form Approved
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1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED
19 June 2000	FINAL Report	1 May 1998 – 30 April 2000

4. TITLE AND SUBTITLE

Expression and Directed Evolution of Peroxidase Enzymes

5a. CONTRACT NUMBER

5b. GRANT NUMBER

N00014-98-1-0657

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Office of Naval Research
800 N. Quincy Street
Arlington, VA 22217-5000

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AILABILITY STATEMENT

DISTRIBUTION UNLIMITED

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The overall goal of this research is to develop strategies for the evolution of novel functions in proteins. The main objective of this project has been to realize the functional expression of eukaryotic peroxidases in facile recombinant hosts (bacteria and yeast), which is a prerequisite to tailoring these enzymes for industrial and Naval applications. We have improved the folding and expression of horseradish peroxidase in recombinant hosts by directed evolution.

15. SUBJECT TERMS

directed evolution, horseradish peroxidase

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Frances H. Arnold
Unclassified	Unclassified	Unclassified	UL	4	19b. TELEPHONE NUMBER *(Include area code)* (626) 395-4162

Standard Form 298 (Rev. 8/98)
Prescribed by ANSI Std. Z39.18